Effect of Cycloheximide on the Development of Thermotolerance and the Synthesis of 68-kilodalton Heat Shock Protein in Chinese Hamster V79 and Mouse L Cells In Vitro

KENZO OHTSUKA1, MASAYO FURUYA2, KAZUMI NITTA3 and EIICHI KANO3

Department of Experimental Radiology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464; Department of Anatomy, School of Medicine, Osaka City University, Abeno-ku, Osaka 545; Department of Experimental Radiology and Health Physics, Fukui Medical University School of Medicine, Matsuoka, Fukui 910-11, Japan

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The relation of heat shock protein (HSP) synthesis and development of thermotolerance was compared in Chinese hamster V79 cells of low, and mouse L cells of high, thermosensitivities. Thermotolerance was induced by step-up heating (42°C, 2 hr and 44°C). Cycloheximide did not inhibit the development of thermotolerance in V79 cells. In contrast, no apparent thermotolerance was induced by step-up heating (42°C, 1 hr and 44°C) in L cells. In V79 cells, as shown by two-dimensional gel electrophoresis, two 68-kilodalton heat shock proteins (68-kDa HSPs) were synthesized constitutively and increased markedly when cells were heated at 42°C for 2 hr. Cycloheximide inhibited more than 90% of the total protein synthesis including the 68-kDa HSPs. In contrast, no 68-kDa HSP was detected in L cells at 37°C. When L cells were heated at 42°C for 1 hr, 68-kDa HSP was detected by fluorography but not by Coomassie blue staining. These results suggest 1) that cells constitutively synthesizing 68-kDa HSP had low thermosensitivity and could be thermotolerant without de novo synthesis of the protein, and 2) that cells not synthesizing 68-kDa HSP had high thermosensitivity and could not be thermotolerant because the cellular 68-kDa HSP content was low even when the protein was newly synthesized.

INTRODUCTION

When mammalian cells are heated, those that survive acquire transient resistance to subsequent heat challenge as shown by an increase in cell survival. This phenomenon is termed thermotolerance1,2). Thermotolerance is a drawback in the use of clinical hyperthermia as a treatment against cancer. Although the molecular mechanism(s) for the development of thermotolerance is not yet fully understood, experimental evidence suggests that protein synthesis

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1大塚健三：愛知県がんセンター研究所放射線部，名古屋市千種区田代町鹿子殿81-1159 〒464
2古家雅代：大阪市立大学医学部解剖学教室，大阪市阿倍野区旭町1 4－54 〒545
3新田和美，加納永一：福井医科大学放射線基礎医学教室，福井県吉田郡松岡町下合月23-3 〒910-11
is necessary for its manifestation\textsuperscript{3}).

When prokaryotic and eukaryotic cells are exposed to heat or to other environmental stresses, they respond by synthesizing a family of proteins, the so-called heat shock proteins (HSPs)\textsuperscript{4}). In mammalian cells, enhanced syntheses of HSPs is closely related to the development of thermotolerance\textsuperscript{5-8)}, but direct evidence for this has not been presented. Recent experiments have suggested the possible dissociation of the synthesis of circa 70-kDa HSP and thermotolerance expression\textsuperscript{9-12}). In the investigation reported here, we compared the relation of 68-kDa HSP synthesis and the development of thermotolerance in Chinese hamster V79 cells of low, and mouse L cells of high, thermosensitivities.

**MATERIALS AND METHODS**

**Cells**

Chinese hamster V79 and mouse L cells were cloned and used in vitro. Both clones were seeded in plastic flasks (Corning, 25 cm\textsuperscript{2}) with loosened screw caps then incubated in a CO\textsubscript{2} incubator as the routine maintenance cultures.

**Culture medium**

Cells were maintained in MLN-15 growth medium, one liter of which contained 730 ml of Eagle's MEM solution (Nissui), 100 ml of NCTC-109 solution (Difco), 20 ml of 2.5% (w/v) lactalbumin hydrolysate solution (Difco) and 150 ml of inactivated bovine serum\textsuperscript{13}).

**Heat and/or cycloheximide treatments**

Exponentially growing cells in monolayers were trypsinized to obtain single cell suspensions. The number of cells were counted with a hemocytometer. In order to yield approximately 100 surviving colonies, cells were diluted serially in graded concentrations with MLN-15 then seeded in culture flasks (Corning, 25 cm\textsuperscript{2}). The seeded cells were incubated at 37°C for 1 day prior to use. The flasks were filled with MLN-15 which had been warmed previously at the chosen temperature. The cells then were heated by immersing the flasks in a water bath whose temperature was controlled within ± 0.05°C. The V79 cells were incubated at 42°C for 2 hr and the L cells for 1 hr. The flasks were transferred to a different water bath preset at 44°C then incubated for periods of up to 60 min. After the scheduled heating, the medium in the flasks was discarded and the cells were refed with fresh MLN-15, after which they were incubated for 7 days at 37°C for the final surviving colony check. We added cycloheximide (CH) to the medium at a final concentration of 5 µg/ml (17.8 µM) immediately prior to the 42°C heating then rinsed it out at the end of the heating to examine its effect on the development of thermotolerance.

**Colony forming assay**

The surviving fractions were obtained from a colony forming unit (CFU) as described elsewhere\textsuperscript{13}). Three replicate flasks were used to determine each survival point and one replicate experiment was done to confirm the results obtained in the previous experiments.
Analysis of HSPs

Cells in the late exponential phase grown in large culture flasks (Corning, 150 cm²) were used. The culture medium was replaced with Leu- and Pre-free Eagle's MEM supplemented with 15% inactivated bovine serum, after which 50 μCi of an [3H] amino acid mixture ([3H]-Leu, [3H]-Lys, [3H]-Phe, [3H]-Pro and [3H]-Tyr, Amersham) was added. The V79 cells then were heated at 42°C for 2 hr and the L cells were for 1 hr. In some of the HSP assays, CHX (5 μg/ml) was added prior to the 42°C heating. After this heating, the cells were rinsed 4 times with cold phosphate-buffered saline (PBS) then harvested by trypsinization and washed with PBS by gentle centrifugation. The resulting cell pellet (circa 1 x 10⁷ cells) was lysed by sonication for 20-30 sec in 150 μl of O'Farrell's isoelectric focusing lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). O'Farrell's method was used for two-dimensional gel electrophoresis. A 30-μl sample of the cell lysate (circa 2 x 10⁶ cells, approximately 300 μg protein) was loaded on the first dimension isoelectric-focusing disc gel (pH 4-6.5). The second dimension was a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a...
Table 1. $T_0$ values$^3$ of heat treatment-survival curves in V79 and L cells (min)

<table>
<thead>
<tr>
<th></th>
<th>$44^\circ$C alone</th>
<th>$42^\circ$C, 2hr → $44^\circ$C</th>
<th>$42^\circ$C, 2 hr + cycloheximide → $44^\circ$C</th>
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<tbody>
<tr>
<td>V79 cells</td>
<td>6.45 ± 0.15</td>
<td>27.6 ± 0.97</td>
<td>25.1 ± 3.9</td>
</tr>
<tr>
<td>L cells</td>
<td>2.10 ± 0.17</td>
<td>1.97 ± 0.46</td>
<td>2.24 ± 0.30</td>
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</table>

a) $T_0$ values show that the heating period required for cell survival was reduced $1/e$ (37%). They were calculated from the survival curves in Figs. 1 and 2 by linear regression analysis.

b) Cycloheximide concentration: 5 µg/ml (17.8 µM).

Fig. 2. Heat sensitivity of L cells at $44^\circ$C and the effect of cycloheximide on it. Cycloheximide (5 µg/ml, 17.8 µM) was added to the medium during heating at $42^\circ$C for 1 hr. Each point is the mean ±1 S. E. (n=6) of two replications.
slab gel with a 10% acrylamide concentration. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Nakarai) then photographed. For fluorography, the stained gels were soaked in 1 M sodium salicylate for 20 min then dried under a vacuum and exposed to X-ray film (Kodak, X-Omat AR5) at -70°C. A 10-µl sample of the cell lysate was precipitated with 10% trichloroacetic acid and its radioactivity was counted in a liquid scintillation counter (Beckman).

RESULTS

The heat treatment period-survival curves of the V79 cells are given in Fig. 1. When the cells were heated at 44°C immediately after heating at 42°C for 2 hr with or without CHX, the slope of the survival curves was markedly reduced (Table 1), evidence of the development of thermotolerance. Heating at 42°C for 2 hr was sufficient for expression of thermotolerance in V79 cells. CHX had no appreciable inhibitory effect on the development of thermotolerance (Fig. 1 and Table 1).

Fig. 3. Two-dimensional gel electrophoresis of the whole cell lysate of V79 cells labeled with a [3H]amino acid mixture. A and A', control cells incubated at 37°C for 2 hr; B and B', cells heated at 42°C for 2 hr; C and C', cells heated at 42°C for 2 hr in the presence of cycloheximide (5 µg/ml). A, B and C, stained with Coomassie blue; A', B' and C', fluorography of the A, B and C gels. Approximately 300 µg protein (2 x 10⁶ cells) was loaded on each first dimension gel. Radioactivities applied to the first dimension gels were 64,284 cpm for A', 69,636 cpm for B' and 5,019 cpm for C'. Exposures to X-ray film were 51 days for A' and 40 for B' and C'. Arrows, 68-kDa HSPs; Ac, actin. The acidic end of the gel is to the right here and in Fig. 4.
L cells were more thermosensitive than V79 cells (Fig. 2). The slope of the survival curve of step-up heating (42°C, 1 hr and 44°C) was similar to that for heating at 44°C alone (Fig. 2, Table 1), evidence of no appreciable development of thermotolerance regardless of the presence of CHX during prior heating at 42°C for 1 hr.

Proteins of the whole cell lysate of V79 cells were separated by two-dimensional gel electrophoresis (Fig. 3). V79 cells constitutively synthesized two 68-kDa HSPs with slightly different isoelectric points (arrows in Fig. 3A and A'). When these were heated at 42°C for 2 hr, there was a considerable synthesis of 68-kDa HSPs, shown by increase in the protein spot sizes (arrows, Fig. 3B and B'). But the spot sizes of other proteins (e.g. actin) were not changed by 42°C heating. No other HSPs were detected under our experimental conditions. When the cells were treated with CHX during 42°C heating, more than 90% of the de novo synthesis of the total proteins, including that of 68-kDa HSPs, was inhibited (Table 2). No fluorographic spots were detected (Fig. 3C).

In contrast, no appreciable amount of 68-kDa HSP was synthesized constitutively by L cells (Fig. 4A and A'). Heating L cells at 42°C for 1 hr resulted in the de novo synthesis of the 68-kDa HSP, as demonstrated by fluorography (arrow, Fig. 4B'), but not seen with Coomassie blue staining (arrow, Fig. 4B). This means that although the 68-kDa HSP was newly synthesized, its amount was so small as to be undetectable by Coomassie blue staining. 30-kDa HSP also was detectable only by fluorography (arrowhead, Fig. 4B'). CHX inhibited more than 90% of the total protein synthesis (Table 2), and no fluorographic spot was found (Fig. 4C'). Possibly, heating at 42°C for 1 hr was not sufficient to induce greater synthesis of HSPs and thermotolerance in L cells. Because L cells were sensitive to heat, they became detached from the culture flask during the 42°C heating and most were lost during the procedures. Therefore, we could not prepare enough cell lysate of longer heating period for the two-dimensional gel electrophoresis.

Table 2. Radioactivity of the acid precipitable fraction of the whole cell lysate of V79 and L cells labeled with [3H]-amino acid mixture (cpm)

<table>
<thead>
<tr>
<th></th>
<th>37°C, 2 hr</th>
<th>37°C, 2 hr + cycloheximide</th>
<th>42°C, 2 hr</th>
<th>42°C, 2 hr + cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79 cells</td>
<td>21,428</td>
<td>1,197</td>
<td>23,212</td>
<td>1,673</td>
</tr>
<tr>
<td>L cells</td>
<td>15,145</td>
<td>1,032</td>
<td>12,186</td>
<td>913</td>
</tr>
</tbody>
</table>

a) - 10 µl of the cell lysate was precipitated with 10% trichloroacetic acid. Radioactivity was counted in a liquid scintillation counter.

b) - Cycloheximide concentration: 5 µg/ml (17.8 µM).
Fig. 4. Two-dimensional gel electrophoresis of the whole cell lysate of L cells labeled with a [3H]-amino acid mixture. A and A’, control cells incubated at 37°C for 1 hr; B and B’, cells heated at 42°C for 1 hr; C and C’, cells heated at 42°C for 1 hr in the presence of cycloheximide (5 µg/ml). A, B and C, gels stained with Coomassie blue; A’, B’ and C’, fluorography of the A, B and C gels. Approximately 300 µg protein (2 x 10⁵ cells) was loaded on each first dimension gel. Radioactivities applied to the first dimension gels were 45,435 cpm for A’, 36,558 cpm for B’ and 2,739 cpm for C’. Exposures to X-ray film were 41 days for A’ and 40 for B’ and C’. Arrow, 68-kDa HSP; arrowhead, 30-kDa HSP; Ac, actin.

DISCUSSION

In V79 cells, CHX inhibited the de novo synthesis of 68-kDa HSPs, but did not prevent the development of thermotolerance induced by step-up heating, whereas, in L cells, 68-kDa HSP was newly synthesized at 42°C in the CHX-free culture medium, but no appreciable thermotolerance was induced by step-up heating. These results indicate that the amounts of newly synthesized HSPs and the development of thermotolerance are not correlated simply. Other evidence of the lack of a simple correlation between the two phenomena have been reported: Landry et. al.⁹) observed that HSP synthesis is not necessarily required for the development of thermotolerance in Morris hepatoma cells. Widelitz et. al.¹⁰) showed that rat fibroblasts adapted (prior heated) at 39°C for 6 hr were thermotolerant but did not synthesize a 68-kDa HSP during subsequent 42°C heating. Burns et. al.¹²) also reported that thermotolerant L1210 murine leukemia cells, induced by a gradual increase in temperature from 37° to 42°C over 180 min, failed to synthesize a 69.5-kDa HSP. Ferrini et. al.¹¹) demonstrated that two human
melanoma cell lines, markedly different in their thermosensitivities, expressed 72-kDa HSP to similar extents when the two lines were heated at 42°C for 60 min.

In contrast, experimental evidence of a positive correlation between HSP synthesis and the development of thermotolerance has been obtained with split heating, in which thermotolerance was induced by an initial (or conditioning) heating at 43 to 46°C for a short period and developed during the following incubation at 37°C. The content of newly synthesized HSPs correlated well with the degree of thermotolerance. The effect of CHX on the development of thermotolerance reported here differs from that reported by Henle and Leeper. They demonstrated that when Chinese hamster ovary cells were exposed to CHX (1 μg/ml) during incubation at 37°C for 7 hr between a conditioning (45°C for 10 min) and second heating, the degree of thermotolerance was approximately half that of the control without CHX.

These experimental results suggest that the molecular mechanism(s) for the development of thermotolerance differs with the inducing conditions used for low hyperthermia (heating below 43°C) and high hyperthermia (heating above 43°C for a short period followed by 37°C incubation for several hours).

The intracellular localization of an approximately 70-kDa HSP has been demonstrated by indirect immunofluorescent staining using polyclonal or monoclonal antibodies against 70-kDa HSP, but the function of the HSP has yet to be determined. When cells were heat-shocked, their nuclei (especially the nucleoli) were stained brightly, the staining pattern diminishing gradually as the cells returned to the normal growth temperature. Diffuse cytoplasmic and nuclear staining, however, was present in cells growing at 37°C. This transient accumulation of 70-kDa HSP in the nuclei and nucleoli was not inhibited by CHX.

Thermotolerance was induced without de novo synthesis of 68-kDa HSPs in V79 cells (Fig. 1 and 3), which suggests that the redistribution of constitutively synthesized 68-kDa HSPs function in the development of thermotolerance. The thermosensitivity of cells without prior heating, under our experimental conditions, appeared to depend on the content of constitutively synthesized 68-kDa HSPs, as shown by Coomassie blue staining.

To obtain details about the relation between HSPs and thermotolerance, we need to measure the total amount of each HSP, the newly synthesized as well as the constitutive type. We are now using rabbit antiserum against the 68-kDa HSP of murine mastocytoma cells to obtain these details.

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REFERENCES


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Figure: Figure 3 should read Figure 4, and Figure 4 should read Figure 3.